Formulation of vegetable fat pellets with pheromone and *Beauveria bassiana* to control the larger grain borer, *Prostephanus truncatus* (Horn)

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Abstract: The use of hydrogenated rapeseed oil as a carrier for conidia of the entomopathogenic fungus *Beauveria bassiana* (Bals) Vuill was investigated as part of a research programme on the control of the larger grain borer, *Prostephanus truncatus* (Horn). Melting the oil, which is solid at temperatures below 32 °C, allows the incorporation of materials such as aggregation pheromones and conidia; sudden cooling produces solid fat pellets.

In attraction tests conducted with pellets containing *P truncatus* aggregation pheromone, significantly higher numbers of beetles were attracted to pellets containing pheromone at a concentration of 4 ml litre^{-1} compared to control pellets for at least four weeks when held in Petri dishes in the laboratory and for at least six weeks when pellets held in insect traps were exposed to outside conditions. The attraction was retained over a period of storage in glass bottles; pellets stored in the freezer or incubator (at −10 °C or 27 °C) attracted beetles according to the pheromone level for the duration of the work (14 and 13 months respectively). When pheromones and conidia were incorporated into the same pellets they could be stored in a freezer or refrigerator retaining over 80% viability after 51 weeks; those stored in an incubator at 27 °C showed significantly lower germination at 20.7–27.2% after the same time. There was an indication that the pheromone caused a slight reduction in the viability of conidia, although this may have been just a slight delay in the speed of germination.

Rapid dose transfer from pellets with conidia with and without the addition of pheromone was demonstrated. Insects were exposed to pellets for 24 hours and 96–100% mortality was observed in treatments containing conidia within six days of exposure.

Keywords: *Beauveria bassiana*; *Prostephanus truncatus*; fat pellets; formulations; conidia; pheromone

1 INTRODUCTION

There have been many recent advances in the use of entomopathogenic fungi, such as the hyphomycetes *Metarhizium* spp and *Beauveria* spp, leading to increased interest in their wider use for the control of insect pests. Much of this is due to the awareness that, for maximum efficacy, advances in formulation and application techniques and knowledge of the ecology of the pest system are integral to success; the active ingredient, the fungal conidia, is not the only important feature in a mycopesticide. Consequently, it is now possible to use fungi in extremely arid conditions,[1] to exploit secondary cycling of the pathogen to achieve long-term control[2] and to maintain viability of conidia where storage temperatures are high.[3]

One area that has received little attention to date is the control of insect pests of stored grains using entomopathogenic fungi. One such pest, the larger grain borer *Prostephanus truncatus* (Horn), was accidentally introduced into East Africa in the late 1970s[4] and into West Africa in the early 1980s[5] and has since been regarded as a devastating pest. For details of distribution and effect see Farrell *et al.*[6] An internationally co-ordinated biocontrol programme resulted in the introduction of the histerid beetle predator *Teretrius (Teretriosoma) nigrescens* Lewis into Kenya.[7] While there are reports that its main effect is in the outside environment rather than in stores, it has been shown to significantly reduce numbers of *P truncatus* in some maize stores. However, the ability of the predator to control the pest in all climatic

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conditions is still uncertain.[7] The need for further control measures, which would also be effective against other pest species, led to a research programme investigating the use of entomopathogenic fungi to control storage pests.

Preliminary work had been conducted with *Metarhizium anisopliae* (Metsch) Sorokin and *Beauveria bassiana* (Bals) Vuill against pests such as *Acanthoscelides obtectus* (Say) and *Sitophilus zeamais* Motsch.[8,9] The latter work demonstrated that non-specific isolates of *Beauveria bassiana* gave good control of *S zeamais* in laboratory bioassays. In addition, high kill was achieved with low doses (500 conidia per insect) of one of the best isolates.[9]

The work described above[9] used dry conidia powder mixed in with the grain. Although mycopesticides are considered to be relatively safe,[10,11] very much so in comparison with chemical pesticides, the presence of dry conidia in foodstuffs is undesirable as they present potential allergenic hazards. While these would be removed during the normal processes of washing and cooking during food preparation, effective formulations that eliminate this potential hazard need to be investigated. Hidalgo *et al*[12] worked with fat pellets made from hydrogenated rapeseed oil, incorporating conidia of *B bassiana* and demonstrating kill of *S zeamais* with an unknown (but extremely low) dose transfer to the insects.

The present work describes the use of the fat pellets[12] as a formulation technique for *Beauveria* sp (probably *bassiana*) to control *P truncatus*. The research examined such aspects as storage properties of the formulated product, the effects of incorporating pheromones specific to *P truncatus* and the efficacy of dose transfer to the target insect.

2 EXPERIMENTAL METHODS

2.1 Fat pellets

Hydrogenated rapeseed oil (Seatons, Hull, UK) was used to prepare pellets by a method described by Hidalgo *et al.*[12] The material starts to melt at 32 °C, clears by 38–40 °C, begins to solidify at 29–32 °C and is solid below 25 °C. Materials such as pheromones or fungal conidia can be added while the material is liquid (40 °C is not harmful to dried conidia[3]). A 5-ml syringe, without needle, was used to drop the liquid fat into a beaker containing 70% alcohol at 5–10 °C, causing rapid cooling and solidifying, usually in a hemispherical shape, occasionally spherical. These pellets ranged in size from 2.5 to 6.6 mm diameter, with a mean of 4.5 mm. The pellets were then washed with tap water and stored at 5–8 °C.

2.2 Pheromone

The components of *P truncatus* aggregation pheromone, Truncall 1(T1; isopropyl (*E*)-2-methylpentenoate) and Truncall 2(T2; isopropyl (2*E*,4*E*)-2,4-dimethyl-2,4-heptadienoate)[13,14] were supplied in separate glass vials containing 250 mg of pheromone. They were stored at 5–8 °C until required. For incorporation into the fat pellets, a known volume of pheromone was added to the melted hydrogenated rapeseed oil at a concentration of 4 ml litre^{-1}. Two serial dilutions produced concentrations of 0.4 and 0.04 ml litre^{-1}. The attraction experiments were conducted with T1 but in the final experiment, on dose transfer, both T1 and T2 were used in separate experiments.

2.3 Fungal isolate

The isolate of *Beauveria bassiana* used was obtained by baiting a soil sample from Kibwesi, Eastern Province, Kenya with larvae of the wax moth *Galleria mellonella* L. The fungus was isolated from the wax moth cadaver and cultured on Saboraud dextrose agar. Conidia were obtained by production under a two-phase system.[15] Fungal biomass was produced in a liquid medium of yeast and glucose followed by solid-phase production of conidia on rice grains. Conidia were harvested from the rice substrate, after air drying, by sieving through a 106-μm mesh. Conidia were stored in sealed containers with dried, non-indicating silica gel[16] in a freezer until required for formulation.

2.4 Attraction arena

Crosses were drawn, in pencil, on five 5-cm filter papers to divide each into four, numbered, quarters; these were placed into the bottom of each of five Petri dishes. A fat pellet was placed in the middle of each quarter, with treatments allocated randomly for each trial. In each Petri dish there was a blank pellet, without pheromone, and one pellet each of the three pheromone (T_1) concentrations, 0.04, 0.4 and 4.0 ml litre^{-1}. Five randomly selected, unsexed, *P truncatus* adults were added to each Petri dish, allowed to settle for one minute and then their positions, in terms of quarter, were recorded each minute for ten minutes to compensate for settling behaviour and random movement. This gave 250 counts, 50 from each plate. The 10 readings of each insect are not independent, so these were totalled give the visits to each quarter over the ten-minute period in each plate. These were totalled in turn over the five Petri dishes to give one figure for each treatment, obtained from the actions of all insects. Each test was repeated five times with new insects giving a total of 1250 records per experiment, effectively summarised as five replicates of the numbers recorded in each of the four treatments.

2.5 Attraction tests

2.5.1 Persistence at room temperature

The pellets were set up in Petri dishes as described above and were left *in situ* in the laboratory in temperatures of approximately 24–25 °C. Attraction was monitored weekly from the day of preparation until no differences were observed in the response of *P truncatus* to the pellets of different concentrations of pheromone.

2.5.2 Persistence in delta traps

Pellets of each concentration were placed in delta traps designed for monitoring *P. truncatus* populations in the field.[17] Five traps containing five pellets of each concentration were suspended outside the laboratory in the prevailing climatic conditions at Muguga, 2100 metres above sea level near Nairobi, Kenya. Diurnal temperatures varied from around 4–6 °C (mean minimum) to 18–22 °C (mean maximum) during the experiment. Pellets were collected immediately prior to the attraction test and placed in the Petri dishes as described above with the first assessment being one week after preparation of the pellets. After the attraction test the pellets were returned to the trap. Attraction tests were carried out weekly between 10 April and 19 June until no differences in attraction were observed.

2.5.3 Persistence stored in glass bottles

Metal-topped glass bottles (28-ml Universal type) were used to store the pellets, one bottle per pheromone concentration stored in a freezer at −10 °C and a similar batch in an incubator at 27 °C. Pellets from the freezer and incubator were assessed separately. Five pellets were removed monthly from each bottle and used in attraction tests. Used pellets were returned to storage in fresh bottles. Fresh pellets were used each month until no new pellets were available then those previously assayed once were re-used. This happened after seven months' storage in the incubator and nine months in the freezer. The attraction of the pellets was assessed monthly for up to 14 months.

2.5.4 Viability of fungal conidia stored in fat pellets or as conidial powder

Four treatments were set up. Pellets were prepared containing fungal conidia with or without pheromone at a concentration of 4 ml litre^{-1}. Each type of pellet was placed in nine McCartney bottles. In addition, 18 bottles of dry conidial powder were prepared, of which nine also received non-indicating silica gel to give two further treatments. Three replicates of each treatment were placed under the following conditions: freezer at −10 °C, refrigerator at 5–8 °C and incubator at 27 °C. Viability of conidia was assessed monthly (until at 73 weeks no pellet samples remained) by removing a small amount of conidia powder or half of one pellet from each bottle and suspending in 10 ml kerosene in a Universal bottle. The kerosene bottles were vortex mixed; those containing pellets were mixed until the pellets had completely dissolved.

An aliquot from each bottle of conidial suspension was added to a Petri dish of Saboraud dextrose agar and spread across the surface of the agar by gently tilting the plate from side to side. Germination counts were made after incubating the plates at 27 °C for 24 h.

2.5.5 Dose transfer of conidia

Five treatments were used; four with fat pellets and one absolute control with nothing added. The main interest was in dose transfer from fat pellets containing conidia, but in addition pheromone component T1 or T2 was added to one treatment to determine its effect on conidial viability and virulence. The four batches of fat pellets were: no additives, conidia and pheromone, conidia alone and pheromone alone. The concentration of conidia in pellets was determined by suspending portions of pellets in 10 ml Shellsol T (a low-viscosity mineral oil) and counting the conidia as above. In the experiment using the T1 pheromone component, the pellets with pheromone contained 7.6×10^9 conidia g^{-1} vegetable fat while those not containing pheromone contained 6.3×10^9 conidia g^{-1}. Although all previous experiments had been conducted with component T1 the opportunity was taken to test the effect on dose transfer of component T2 in a separate experiment. Pellets with pheromone component T2 contained 5.6×10^9 conidia g^{-1} while the corresponding pellets without pheromone had 5.7×10^9 conidia g^{-1}. Viability of the conidia in the pellets was over 98% after 24 h. The pellets were stored in the refrigerator for two weeks after production until required for experimental work; they retained full viability. One gram of pellets (about 35 pellets each of 0.026–0.029 g) was placed in a 5-cm Petri dish. Four replicate groups of 25 *P truncatus* per treatment were set up. The insects were introduced into the dish which was sealed with Parafilm. The insects were removed from the dish after 24 h and transferred to a 7.5×2.5 cm glass tube with a perforated top, half filled with ground maize. Mortality was assessed daily for 10 days after treatment. Dead insects were surface-sterilised by washing in 5% sodium hypochlorite followed by three washes of sterile distilled water. The cadavers were placed on damp filter paper in Petri dishes to promote growth of *Beauveria* sp on infected insects.

The experiment using the T2 pheromone component was carried out in an incubator at 27 °C, whereas the experiment using T1 component was carried out at the Kiboko station of the Kenya Agriculture Research Institute (KARI) under ambient conditions.

3 RESULTS

3.1 Attraction tests

Results are given in Figs 1–4; Chi-squared analysis was carried out to test the null hypothesis that the treatments were of equal attractiveness, so the expected value for all treatments was one-quarter of grand total. A significant result indicates non-random distribution of insects between the four treatments. The Chi-squared tests were conducted on results from the beginning, middle and end of each experiment as an *a priori* decision (Table 1). Conducting the test for every sampling occasion would greatly increase the risk of falsely rejecting the null hypothesis of random distribution (Type 1 Error).

Error bars were not applied as the analyses were

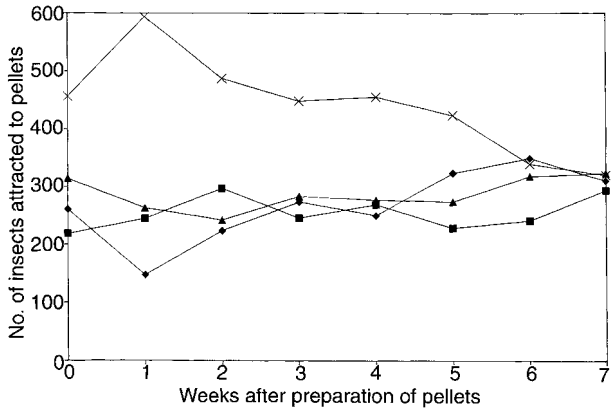

Figure 1. Attraction of *Prostephanus truncatus* to pellets containing different concentrations of pheromones. The pellets remained under laboratory conditions for the duration of the experiment. -◆- Control; pheromone at -×- 4.0, -▲- 0.4 and -■- 0.04 ml litre^{-1}.

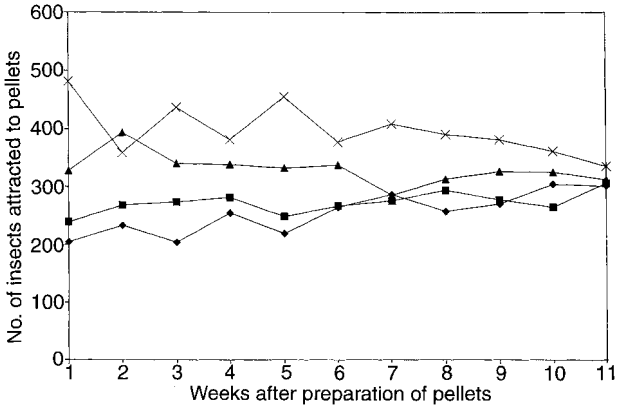

Figure 2. Attraction of *Prostephanus truncatus* to pellets containing different concentrations of pheromones. The pellets remained in delta traps outside the laboratory for the duration of the experiment. -◆- Control; pheromone at -×- 4.0, -▲- 0.4 and -■- 0.04 ml litre^{-1}.

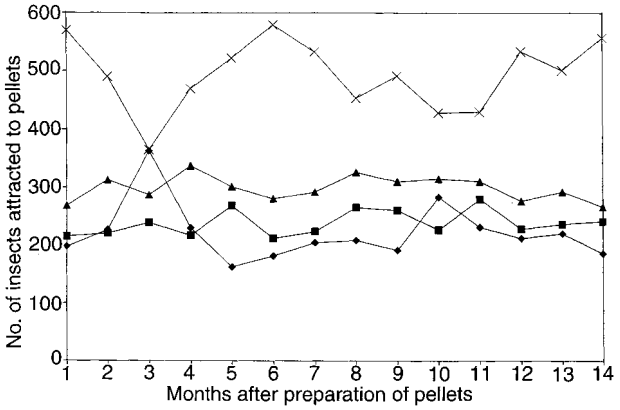

Figure 3. Attraction of *Prostephanus truncatus* to pellets containing different concentrations of pheromones. The pellets were stored in a freezer at −10°C for the duration of the experiment. -◆- Control; pheromone at -×- 4.0, -▲- 0.4 and -■- 0.04 ml litre^{-1}.

conducted on total insect numbers as shown in the graphs. Calculation of means and 95% CI was not deemed appropriate for the experiment.

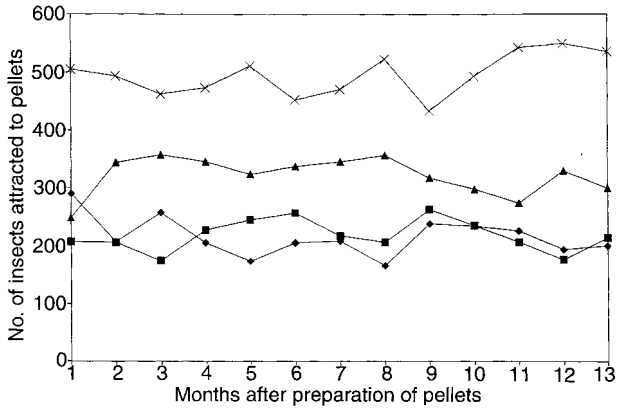

Figure 4. Attraction of *Prostephanus truncatus* to pellets containing different concentrations of pheromones. The pellets were stored in an incubator at 27°C for the duration of the experiment. -◆- Control; pheromone at -×- 4.0, -▲- 0.4 and -■- 0.4 ml litre^{-1}.

Table 1. Analysis of attraction tests at three assessments during the course of experiments

Storage conditions	*Time*	*Chi-square value*	*Probability P=*
Laboratory bench	0 week	16.6	<0.002
	3 weeks	68.2	<0.001
	7 weeks	2.4	<0.5 n.s.
Delta traps (outside)	1 week	143.1	<0.001
	6 weeks	30.3	<0.001
	11 weeks	2.1	>0.5 n.s.
Freezer −10°C	1 month	391.3	<0.001
	7 months	225.2	<0.001
	13 months	162.4	<0.001
Incubator 27°C	1 month	169.1	<0.001
	7 months	157.3	<0.001
	13 months	231.9	<0.001

3.1.1 Attraction of pellets held at room temperature

The pellets containing 4.0 ml litre^{-1} pheromone attracted significantly more *P truncatus* than control pellets or pellets with lower concentrations of pheromone for at least four weeks (Fig 1). By eight weeks there was no evidence of non-random distribution of the insects, indicating no difference in attraction by the control and the highest dose of pheromone treatment.

3.1.2. Attraction of pellets from delta traps

Beetles were attracted to the pellets generally according to concentration (Fig 2). Most beetles were attracted to the highest pheromone concentration at all but one assessment, and higher numbers of beetles were attracted to the 0.4 ml litre^{-1} than to the the 0.04 ml litre^{-1} concentration at each of the 11 sampling dates. At weeks 1 and 6 there were significant departures from random distributions, indicating differences in attraction.

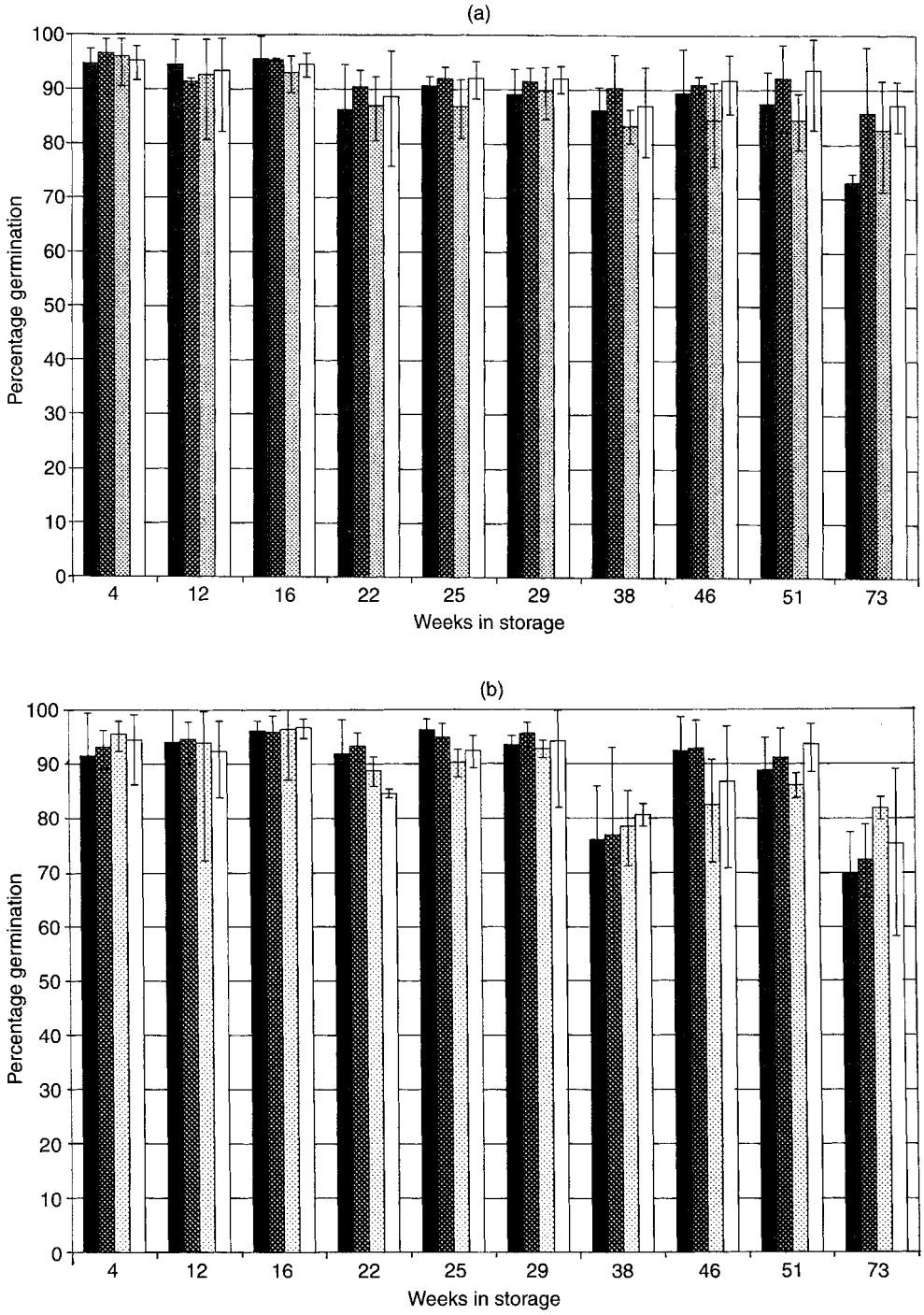

Figure 5. Germination of conidia of *Beauveria bassiana* stored in (a) the freezer, (b) the refrigerator and (c) the incubator (over page). (■) Pellets plus conidia plus pheromone, (▒) pellets plus conidia, (⬚) conidia stored as powder, (□) conidia stored as powder plus silica gel. Error bars show 95% Confidence Intervals.

3.1.3 Attraction of pellets contained in glass bottles

Beetles were attracted to the pellets according to pheromone concentration for the duration of the experiment in both freezer and incubator (Figs 3 and 4; Table 1). The numbers of beetles attracted to control pellets were comparable with those attracted to pellets containing the lowest concentration of pheromone from the incubator test.

3.1.4 Viability of fungal spores stored in fat pellets or as conidial powder

Results are given in Figs 5a, b and c showing germination of conidia with 95% confidence intervals calculated using arcsine transformed data. There was no consistent evidence of statistically significant differences between any of the treatments at any one sampling date. Apparent differences at, for example, week 22 in the refrigerator treatment were not present at week 25. At week 73 the viability of conidia mixed with pheromone and pellet in the freezer may have been reduced compared with that of the conidia plus silica gel treatment. Similarly, judged from CIs, pelleted conidia in the fridge may have lost viability compared with conidia stored as powder (Figs 5a and

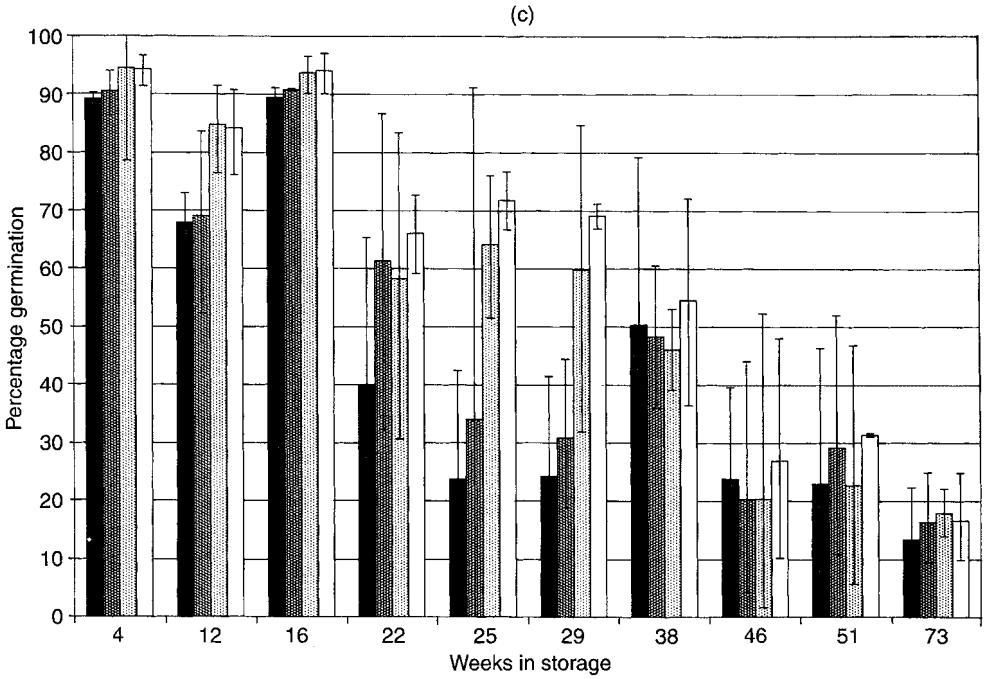

Figure 5. Continued.

b). However, without subsequent samplings these differences should not be viewed as either biologically or statistically significant. Of real importance was that, after 51 weeks in storage, germination ranged from 84.5 to 93.6% in the freezer treatments and 86.1 to 93.6% in the refrigerator. Even at week 73 viability was over 70% in the treatment incorporating conidia and pheromone into pellets and over 80% in the other treatments.

The material stored in the incubator showed significantly lower germination than that in the freezer and refrigerator, in all treatments, with germination below 30% at week 46 and ranging between 22.7 and 31.4% by week 51 and 13.4 and 17.9% by week 73 (Fig 5c). A characteristic of fungal conidia is that viability is often maintained at a steady level for a period and then there is a very rapid decline.[3] Replicates of a treatment often reflect this, showing marked differences in germination, resulting in large error bars. The presence of such large error bars is both a strong indication of a real effect and a feature that makes the demonstration of statistically significant differences difficult. The data suggest that biologically significant declines in viability began after week 16. Generally germination levels appeared higher in the conidial powder treatments than in the pellets, to judge from the incubator results. Overall there was also an indication that the addition of silica gel was beneficial for retaining viability of the conidia compared with conidia alone. The addition of silica gel resulted in higher germination in 22 of the 27 sampling occasions and of the five sampling occasions where the conidial powder alone was higher, none occurred after week 25.

Although there were no statistically significant differences between germination of conidia from pellets with or without pheromone, germination was higher in the pellets without pheromone at 21 sampling occasions and lower in only five (one equal).

3.1.5 Dose transfer

Figures 6a and b show the cumulative percentage mortalities observed for the 10 days following treatment. Mortalities of the absolute controls ranged from 1 to 7%, while those of pellets without conidia were 2–20% by day 10, with no indication that the inclusion of pheromone into pellets had a mortality effect on *P truncatus*. By contrast, in the pellet treatments containing conidia, 100% mortality was achieved by day 6 in one experiment (Fig 6a) and 96% mortality by day 4 in the other experiment (Fig 6b). For the pellets containing pheromone component T1 the peak of infection was a day later than in the treatment without pheromone. There was no difference between pellets containing conidia with or without pheromone component T2. Only cadavers from the treatments with conidia showed the sporulation characteristic of *Beauveria* sp.

4 DISCUSSION AND CONCLUSIONS

The objective of the work was to formulate conidia of *Beauveria bassiana* such that no airborne conidia, which may be a potential allergen hazard, would be present in grain stores. Concentrating the conidia in pellets means that the beetles need to come in contact with the pellets to receive a dose and aggregation pheromone was added to pellets in an attempt to attract *P truncatus*. To be effective, this attraction would need to be very localised so as not to attract the

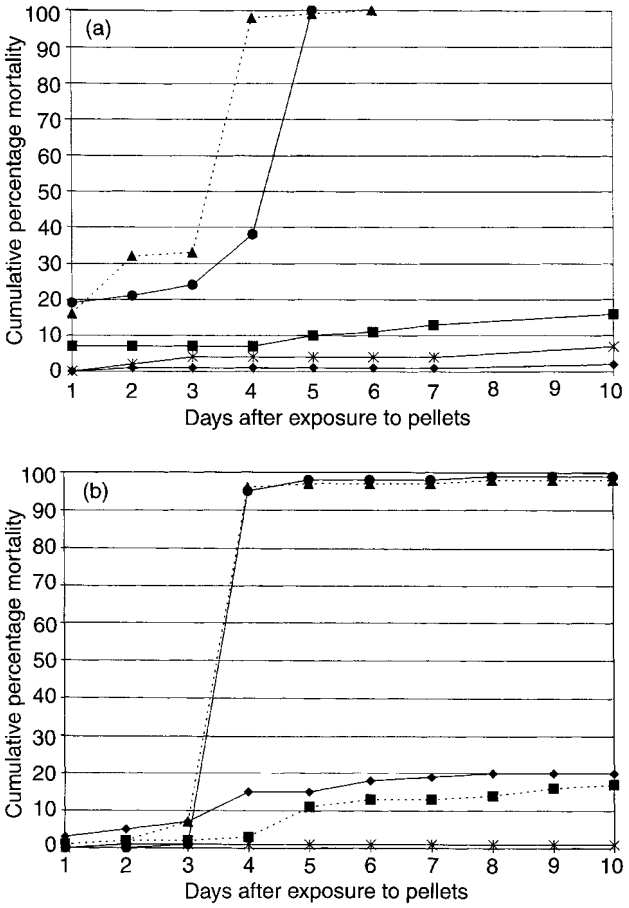

Figure 6. Cumulative mortality of *Prostephanus truncatus* exposed to (-◆-) fat pellets plus pheromone (-■-), fat pellets plus conidia of *Beauveria bassiana*, (-▲-) fat pellets plus pheromone plus conidia of *Beauveria bassiana* or to (-×-) absolute control. Figure 6a shows the experiment conducted using pheromone component T1 and Figure 6b using pheromone component T2.

beetles into the store; the intention was that beetles already in the store and still responsive to the pheromone would move towards the pellets and pick up a lethal dose of conidia. Whether this attraction can be confined to a small distance, ie to attract only beetles within the store, rather than to attract them in from outside, may be doubtful.[18,19] This would require investigating if it was shown to be feasible to incorporate pheromone and conidia within the fat pellets, to store them without loss of efficacy for significant periods and to demonstrate dose transfer.

The formulated pellets demonstrated reasonable retention of attraction due to pheromone T1, storage of conidia (under cold conditions) of over a year in pellets both with and without pheromone and good dose-transfer characteristics. The last feature was exhibited by pellets both with and without pheromone. Dose transfer from the pellets produced very high levels of kill; the dose was not measured but was probably very low as the pellets maintained their integrity over the 24 h of exposure to the insects. The fat may have assisted in sticking the conidia onto the insects, overcoming the first line of defence of the insect which involves preventing adhesion of conidia. However, the insects were exposed to large numbers of pellets for significant periods of time. Having established the basic features of the formulation, more detailed work is required to determine if the pheromone(s) can be of practical value. This must take into account recent research such as that demonstrating properties of the constituents and differential responses of the sexes and the benefits of 1:1 mixtures of T1 and T2 utilising the different properties of each constituent.[20]

Once prepared, the pellets could be stored, or used immediately. Pellets containing 4.0 ml litre^{-1} pheromone attracted significantly higher numbers of *P truncatus* after four to six weeks of exposure in the laboratory or field. If this attractiveness resulted in increased dose transfer for that period of time, without attracting more insects from outside the stores, this could give protection against beetles migrating to stores and some of their progeny. Mycopesticides have an advantage over chemical pesticides in that they can recycle; when infected insects die they sporulate, setting up foci of further infectivity.[2] The delay to death, often at least four days, gives time for beetles to distribute more widely in the store and if death occurs before eggs are laid, could have the consequence of only minimal grain damage. Consequently, even with a system where conidia are placed in discrete areas in stores, the pathogen would probably soon be more widely distributed through the store.

An effective mycopesticide formulation must have adequate storage characteristics. Various periods have been proposed but 12–18 months is widely advocated.[21] The pellet formulations were stored for 51 weeks in a refrigerator or freezer with minimal loss of conidial viability, although for only a few months at 27 °C. The results show poorer storage at 27 °C than expected from previous work,[3] improvements in drying procedures should extend storage at this temperature. The pheromone remained active for at least 14 months while stored in glass bottles in the freezer and 13 months in the incubator. With a predictable system such as grain storage in East Africa, longer periods of storage should be unnecessary. Protection, whether by chemical, mycopesticide, botanicals or ash has to be added as the stores are filled, after the grain harvest. Therefore, it is predictable, to within a matter of a few weeks, when the pesticide will be required and production planned accordingly.

The health hazards associated with (particulate) conidia in food stores are probably low, as there are few adverse reports on the use of entomopathogens such as *B bassiana*,[11,12] other than the occasional allergenic response usually associated with exposure to large quantities of conidia in eg a mass-production facility. These risks are also relative and are generally less than those presented by chemical pesticides where many adverse reactions have been noted. The risks of adding conidia of *Beauveria* may be less than those associated with insect infestation, which results in

much particulate detritus which may be allergenic,[22] and spoiled grain which often carries a massive dose of fungal spores, including those pathogenic to humans, such as *Aspergillus* spp.

The fat pellets exhibited some properties which could make them a valuable formulation material for a range of insect pests. Further tests are on-going to determine efficacy in typical storage conditions against *P truncatus* and other major species, especially *Sitophilus zeamais*.

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